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Purification and Characterization of Streptococcal Proliferative Factor

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Group A streptococcal infections are often associated with scarlet fever and flares of guttate psoriasis. Previous investigation has demonstrated the presence of a factor in streptococcal culture filtrates capable of stimulating proliferation of rabbit keratinocytes *in vivo* and human lymphocytes *in vitro*. This report outlines an *in vivo* method for the production of streptococcal proliferation factor, its purification, and characterization of its physical properties.

We cultured Group A streptococci (Type 12, Strain NY5) in synthetic media by *in vivo* incubation within dialysis casing surgically implanted in rabbit peritoneum. Streptococcal exoproteins were isolated by centrifugation of the bacteria and millipore filtration. Purification of streptococcal proliferative factor was accomplished by differential solubility and molecular sieve chromatography. No proteolytic or hemolytic activity was discovered in the resulting product. The relative molecular weight of this factor is 29,000 as determined by SDS gels and molecular sieve chromatography. The sedimentation coefficient determined by sucrose gradient ultracentrifugation is 2.7S. Isoelectric focusing showed minimal microheterogeneity with the pI of the major band being 5.0.

Thus, streptococcal proliferative factor can be produced by *in vivo* incubation of streptococci in synthetic media. Purification entails a rapid 2-step process. The relative molecular weight, sedimentation coefficient and isoelectric points have been established.

Group A streptococcal infections of the upper respiratory tract have been related to flares of guttate psoriasis and the exanthem of scarlet fever since the early part of this century [1-5]. The morphologic suggestion of a hyperproliferative response in these conditions led Cole and Wuepper to the discovery of a streptococcal proliferative factor in crude, cell-free culture filtrates from *Streptococcus pyogenes*, Strain NY5. This factor is capable of stimulating the proliferation of rabbit keratinocytes *in vivo* and human mononuclear leukocytes *in vitro* [6].

We now report an *in vivo* technique for the production of this streptococcal proliferative factor (SPF) in a defined, elemental media. Isolation of SPF can be performed by a rapid, 2-step purification. SPF migrates as a single band in SDS polyacrylamide electrophoresis. The relative molecular weight as determined by SDS gels and molecular sieve chromatography is 29,000. The behavior of SPF in sucrose gradient ultracentrifugation and isoelectric focusing has been determined.

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Abbreviations:
FH: Ficoll Hypaque
MNC: mononuclear cells
SPF: streptococcal proliferative factor

MATERIALS AND METHODS

Bacteria

Streptococcus pyogenes, Type 12 (Strain NY5), was used for production of extracellular streptococcal products. This strain was purchased from the American Type Culture Collection.

Exoprotein Production

Bacteria were initially grown from stock cultures on blood agar plates. Colonies were innoculated into 100 milliliter aliquots of RPMI Medium 1640 (GIBCO) contained within low molecular weight pore size dialysis casings (Union Carbide, Chicago, IL). These casings were placed surgically within the peritoneal cavity of New Zealand white rabbits obtained from a local supplier. Casings were allowed to remain *in vivo* for 24 hr and were then surgically removed. Bacteria were removed from extracellular products by centrifugation followed by 0.22 μ millipore filtration. Exotoxin was purified by differential solubility in that it is precipitated by 75% ethanol and is resolubilized in .05 M acetate buffered saline [6,7].

Isolation of Mononuclear Cells (MNC)

Peripheral venous blood samples were collected from healthy adult volunteer donors and layered over Ficoll Hypaque (FH) (Pharmacia) after a 1:1 dilution with saline [8]. Centrifugation at 400 g for 30 min allowed isolation of MNC at the density interface. Cells were aspirated and washed 2 or 3 times with normal saline. The final MNC suspension was carried out in RPMI 1640 supplemented with glutamine, antibiotics, and antimycotics (Gibco). Human AB serum was added at 15%.

MNC Assay for Proliferative Activity

MNC were cultured at 1×10^6 cells per ml. 200 μl aliquots were placed within microtiter plates and cultured in a humidified, 5% CO_2 incubator at 37°C. Stimulated cells were harvested 4 to 5 days later. To each well was added, 16 hr prior to harvest, 10 μl of ^{3}HTdr (New England Nuclear) diluted to 50 μCi/cc. Harvesting was done with a MASH 2 harvester (Microbiological Associates). ^{3}HTdr uptake was determined by liquid scintillation counting using a 1:1 aquasol-toluene mixture.

Polyacrylamide Gel Electrophoresis

Alkaline disc gel electrophoresis was performed according to the method described by Davis [9]. Sodium dodecyl sulfate gel electrophoresis for molecular weight determination was performed as described [10]. Proteins of known molecular weight served as standards. These included phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin (Pharmacia). Gels were stained with Coomasie Blue R-250 (Sigma). Analysis for the presence of carbohydrate was performed by staining gels with periodic acid fuschin stain [11].

Column Chromatography

Gel filtration chromatography was performed in a 1.5×90 cm glass column (Pharmacia) using Ultrogel ACA 54 (LKB). 25 mM ammonium acetate buffer, pH 8.0, served as the eluent. The usual sample application volume was 1.5 cc.

Isoelectric Focusing

Thin-layer polyacrylamide isoelectric focusing was performed using LKB PAGplates with an ampholyte range of 3.5-9.5. The procedure was run on an LKB Multiphor electrophoresis apparatus with an LKB power supply (LKB-Produkten, Stockholm, Sweden). Each run was performed using half PAGplates at constant power of 12.5 w for a period of 1 to 2 hr.

Sucrose Gradient Ultracentrifugation

A 5 to 25% sucrose gradient in ammonium acetate buffer, pH 8.0, was prepared using a Buchler gradient mixer (Buchler Instruments, Fort Lee, NJ). SPF and standards dissolved in ammonium acetate buffer were applied to gradients in 200 μl volumes. Rabbit IgG, bovine serum albumin, and cytochrome c served as standards [12]. Ultracentrifugation was carried out for 18 hr at 50,000 RPM using a Beckman L5-50B preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). Gradient fractions of 10 drop aliquots were assayed by absorption at 230 nm and lymphocyte transforming ability.

Detection of Proteolytic and Hemolytic Activities

Assessment of proteolytic activity in purified preparations of streptococcal proliferative factor was carried out using a casein plate assay [13]. Alteration of the technique with the use of blood agar plates rather than casein plates allowed a qualitative evaluation of hemolytic activity.

RESULTS

Preparation of SPF

Initial experiments with cultures of *Streptococcus pyogenes*, Type 12 (Strain NY5) showed that this organism grew poorly in a protein-free, defined media such as RPMI 1640. Medium 199 and McCoys media also supported only meager growth. Incubation of cultures under high carbon dioxide and low oxygen tensions did not improve bacterial growth.

Modified *in vivo* culture conditions provided an environment within which the streptococcus flourished (Fig 1). RPMI 1640 innoculated with streptococci and placed within dialysis tubing yielded a milky white fluid upon recovery from intraperitoneal incubation for 24 hr. Gram stain of this fluid revealed sheets of gram positive cocci. Streptococci were easily removed from supernatants by centrifugation and millipore filtration. Precipitation in ethanol and resolubilization in acetate buffered saline allowed isolation of SPF.

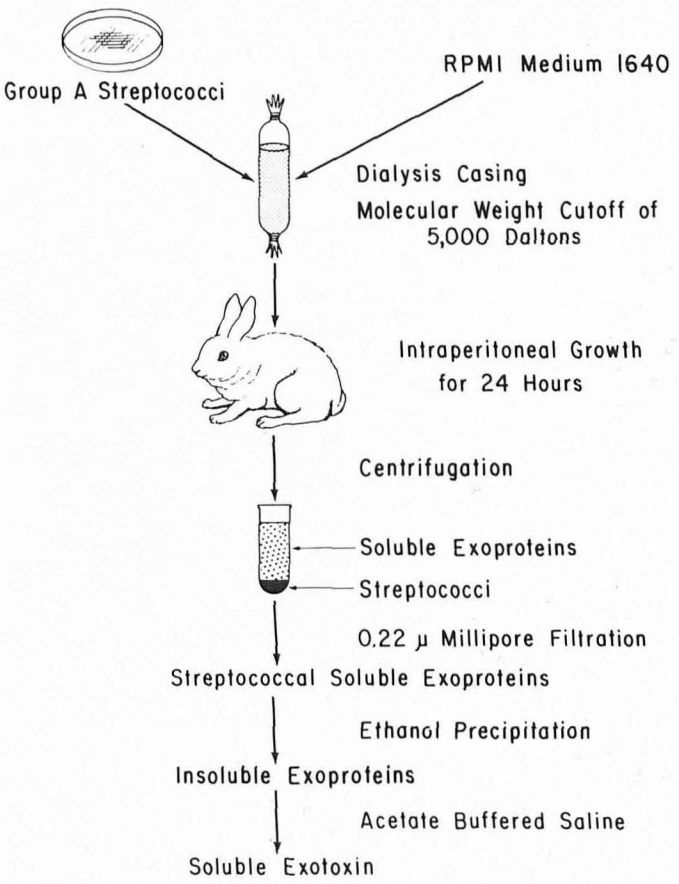

FIG 1. Preparative sequence for the production of streptococcal proliferative factor.

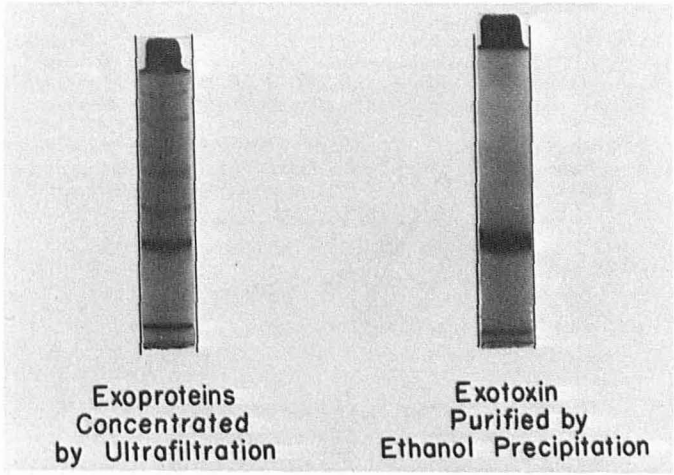

FIG 2. Alkaline disc gel electrophoresis of streptococcal extracellular proteins. Exoproteins concentrated by Amicon UM2 ultrafiltration are shown in the gel on the left. The gel on the right demonstrates streptococcal proliferative factor purified by ethanol precipitation and resolubilization in acetate buffer.

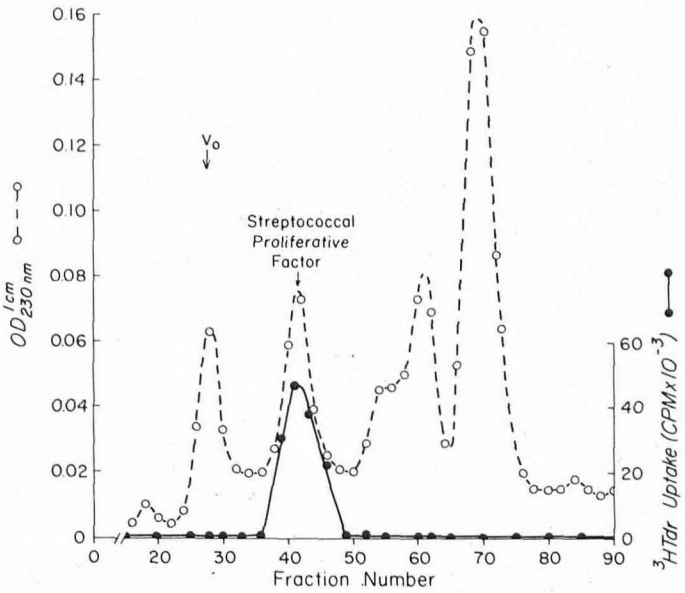

FIG 3. ACA 54 gel filtration chromatography of streptococcal exoproteins in ethanol and acetate buffered saline. Column fractions are assayed for proliferative activity by the mononuclear cell uptake of tritiated thymidine 4 days after stimulation with selected fractions diluted 1:100. Proliferative activity is seen limited to streptococcal proliferative factor.

Analysis of purity by alkaline disc gel electrophoresis is seen in Fig 2. Purified SPF is seen as a single band in contrast to concentrated streptococcal exoproteins prior to purification by differential solubility. The degree of isolation from other streptoccal exoproteins by ethanol precipitation is variable and contaminants not seen in this experiment may appear. Alkaline disc gels of purified SPF failed to show glycoproteins when stained with periodic acid fuchsin stain.

No proteolytic activity was detected qualitatively by the casein plate diffusion assay. Hemolytic activity was absent on blood agar plates.

ACA 54 gel filtration chromatography of a similarly prepared exotoxin is seen in Fig 3. Measurement of optical density at 230

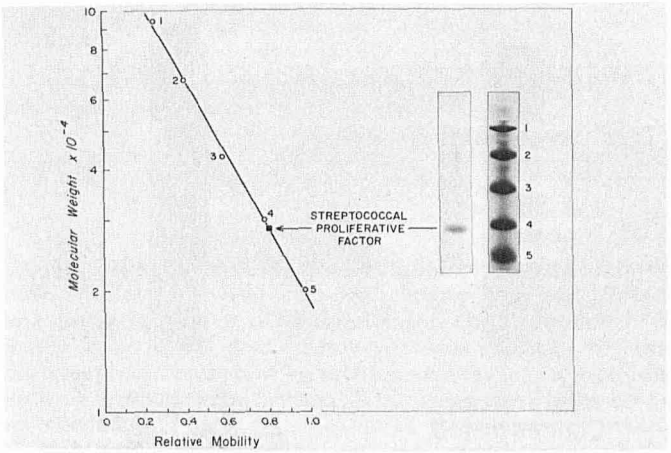

FIG 4. SDS polyacrylamide gel electrophoresis of fraction 41 from ACA 54 gel filtration chromatography. The relative molecular weight of streptococcal proliferative factor by this methoid is 28,500. Protein standards are (*1*) phosphorylase B, (*2*) bovine serum albumin, (*3*) ovalbumin, (*4*) carbonic anhydrase, and (*5*) soybean trypsin inhibitor.

μm reveals the presence of at least 4 protein peaks. When representative column fractions are diluted 1:100 and tested by using a MNC stimulation assay, proliferative activity was detected in fractions 38 through 47. The initial protein peak probably represents higher molecular weight streptococcal products appearing in the void volume, the later peaks are very low molecular weight streptococcal products. The amounts of non-SPF exoproteins seen in exotoxin purified by differential solubility varies from preparation to preparation.

SDS polyacrylamide gel electrophoresis performed on fraction 41 of the ACA 54 gel filtration separation reveals the presence of a single band (Fig 4). The relative molecular weight is 28,500 as compared with known standards. This is in close agreement with the figure of 29,500 obtained by gel filtration on a calibrated column of ACA 54. Treatment with 2-mercaptoethanol does not reveal the presence of subunits.

Sucrose gradient ultracentrifugation in a 5 to 25% gradient revealed SPF to have a $S_{20,\omega}$ of 2.7S. There appeared to be some microheterogeneity upon isoelectric focusing with a prominent band at pI 5.0 and a faint band at pI 4.9.

DISCUSSION

Previous studies investigating the activities of streptococcal exotoxins have utilized standard media such as trypticase soy broth [6], beef heart medium [14], Stock's medium [7], and Todd-Hewitt broth [15]. These media consist of a heterogeneous mixture of proteins, some of them containing proteases, which must be removed from exotoxin preparations or accounted for with special broth controls when performing tests of exotoxin preparations or accounted for with special broth controls when performing tests of exotoxin bioactivity. In studying the functional activity of SPF, we wanted to purify and characterize the exotoxin free of contaminating proteins or oligopeptide fragments.

Attempts at culturing streptococci *in vitro* in synthetic tissue culture media were minimally successful even in conditions of low oxygen tension. We felt that the fastidious nature of the streptococcus could be accommodated using modified *in vivo* conditions. Culture of streptococci within the rabbit peritoneum was successful using dialysis casing with a molecular pore size of approximately 5,000. Essential nutrients, as yet unstudied, are presumed to diffuse into the casing to enhance growth, while bacterial exoproteins are retained within the casings. Although this is an artificial system, it may closely resemble *in vivo* human infections. Should the external milieu of the streptococcus be important in the synthesis of various bacterial products, one would expect similar protein products in both systems. The significance of the external milieu in bacterial exotoxin production has been shown in other systems [16].

The purification of SPF from some supernatant exoproteins was easily performed by differential solubility in ethanol and acetate buffered saline. The results of initial studies suggested that exotoxin could be isolated solely by this step, but further experiments revealed variable amounts of non-SPF exoproteins. ACA 54 gel filtration allowed isolation of SPF as a pure substance. Assay of column fractions for proliferative activity by MNC stimulations identified SPF as the only protein with proliferative capabilities. This 2-step purification is a simple, rapid technique for the isolation of SPF from other streptococcal products.

The average relative molecular weight of SPF is 29,000 by SDS polyacrylamide electrophoresis and ACA 54 chromatography. Nauciel purified an erythrogenic toxin with a molecular weight of 30,500 [17,18]. The characteristics of the MNC response to our exotoxin is similar to those noted by Nauciel (unpublished observations). The work of Watson et al [14,19] with streptococcal pyrogenic exotoxins (erythrogenic toxins) have classified three types of toxins: A, B, and C. They are immunochemically distinct with relative molecular weights of 8,000, 17,500 and 13,000 respectively. Their respective pIs are 4.5–5.5, 8.5–9.5 and 6.7. These substances have also been reported to be mitogenic. The studies of Seravilli and Taranta have explored lymphocyte transformation by Group A streptococcal filtrates. They have found the factor responsible for this property to be a substance with a molecular weight of 26,000 [15]. The exact relationship of SPF to the erythrogenic toxin(s) and the above streptococcal preparations is unclear and needs further study.

In summary, SPF has been produced by Strain NY5 streptococci using a new *in vivo* system of exotoxin production. Purification of this exotoxin is carried out rapidly and easily by differential solubility and ACA 54 chromatography. Under these conditions, SPF is the only exoprotein isolated with proliferative activity. The average relative molecular weight is 29,000. The sedimentation coefficient has been determined to be 2.7S, and the isoelectric point of the major band is 5.0. Further evaluation and characterization of the mechanism of its proliferative activity is in progress.

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Announcement

Eligibility for examination for special competence in dermatopathology: Diplomates of the American Board of Dermatology and the American Board of Pathology who qualify under the "experience" category to take the examination for special competence in dermatopathology must apply before December 31, 1981. Applications cannot be accepted after that date; in order to qualify for the examination in the "experience" category, the candidate must have completed the training requirements for basic certification before December 31, 1977.

Diplomates of the American Board of Dermatology who completed their training before December 31, 1977 and thus qualify for application to take the examination should write to Clarence S. Livingood, M.D., Executive Director, The American Board of Dermatology, Henry Ford Hospital, Detroit, Michigan 48202. It is emphasized that this *must* be done before December 31, 1981.